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Video Article

A Method to Test the Effect of Environmental Cues on Mating Behavior in *Drosophila melanogaster*

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Abstract

An individual's sexual drive is influenced by genotype, experience and environmental conditions. How these factors interact to modulate sexual behaviors remains poorly understood. In *Drosophila melanogaster*, environmental cues, such as food availability, affect mating activity offering a tractable system to investigate the mechanisms modulating sexual behavior. In *D. melanogaster*, environmental cues are often sensed via the chemosensory gustatory and olfactory systems. Here, we present a method to test the effect of environmental chemical cues on mating behavior. The assay consists of a small mating arena containing food medium and a mating couple. The mating frequency for each couple is continuously monitored for 24 h. Here we present the applicability of this assay to test environmental compounds from an external source through a pressurized air system as well as manipulation of the environmental components directly in the mating arena. The use of a pressurized air system is especially useful to test the effect of very volatile compounds, while manipulating components directly in the mating arena can be of value to ascertain a compound's presence. This assay can be adapted to answer questions about the influence of genetic and environmental cues on mating behavior and fecundity as well as other male and female reproductive behaviors.

Video Link

The video component of this article can be found at https://www.jove.com/video/55690/

Introduction

Reproductive behaviors typically have high energy costs, especially for females, who produce larger gametes than males and must carefully chose the conditions to raise their developing offspring. Because of the energy cost, it is not surprising that reproduction is connected to nutritional conditions. This is true in most, if not all, animals including mammals, whose puberty can be delayed by malnutrition, and whose sexual drive can be negatively affected by food-restriction[1].

The reproduction of the genetic model organism *Drosophila melanogaster* is also affected by nutritional conditions. Males court at higher level in the presence of food volatiles[2], and females are more sexually receptive in the presence of yeast, a major nutrient for egg production and offspring survival[3,4,5]. This evolutionary conserved reproductive response to food offers the opportunity to study mechanisms that connect environmental food availability to sexual reproduction in a genetically tractable and time-efficient organism. Indeed, work in *D. melanogaster* has implicated the insulin pathway as an important regulator of the connection between food and mating behavior[6]. It has also shown that the act of mating itself changes the food preference of females as well as the associated chemosensory neurons[7,8,9].

It is clear that food cues affect reproductive behaviors in *D. melanogaster*. These effects seem to mainly affect females, specifically those who have already mated[5]. However, to test these acute effects of environmental conditions the assay classically used for female mating behavior might not be very suitable due to the long interruptions between mating episodes. In the classic remating assay, a virgin female first mates with a male, and is immediately isolated and presented with a new male 24 to 48 h later. This classic assay has been used with great success to identify components of the male ejaculate that modify the female behavior and the female response[12,13,14,15,16,17,18]. The continuous mating assay demonstrated here, is therefore, an addition to classic mating assays that can be used to study the acute effect of environmental conditions on reproductive behaviors.

Using the continuous assay for mating behavior that is explained here, we previously showed that a pair of flies exposed to yeast will remate several times over a 24 h observation period[5,19,20,21], while flies not exposed to food will only remate once[5]. This finding can be puzzling in the light of a large portion of the *D. melanogaster* literature indicating that females do not remate for several days after an initial mating (reviewed in references[10,11]). However, this discrepancy can easily be explained by assay conditions, where a female is isolated for one to several days before a new mating opportunity is provided. If the pair does not mate in this hour-long observation period, the female is characterized as not receptive. Moreover, the high mating frequency should not be surprising given that the data from wild-caught flies show that females contain sperm from 4 to 6 males in their storage organs; thus indicating that females naturally remate several times[22,23].

Here, we demonstrate the use of this continuous mating assay to unravel how flies gather and combine information about environmental conditions to modulate mating frequency. This assay allows one to test a relatively large number of mating couples for genetic studies and to test the influence of volatile and non-volatile environmental cues. The assay typically runs for 24 h, but can be extended to 48 h, allowing the testing of cycling environmental cues such as the light-dark (LD) cycle. We demonstrate this assay by testing the influence of volatile cues from a yeast culture within a pressurized air system in combination with the availability of non-volatile yeast nutrient in the food substrate.

The pressurized air system continuously pumps volatile cues into a mating arena that contains a food substrate and a test couple (whose mating behavior is monitored). To further determine the specifics through which yeast influences mating, we test a major volatile compound of yeast, namely acetic acid[24], in combination with an amino acid content that corresponds to that of yeast in the food substrate, in the form of peptone (amino acids derived from enzymatic digestion of animal proteins). Together these experiments demonstrate how the effect of environmental cues on the mating behavior of *D. melanogaster* can be tested with this assay.

Protocol

1. Environmentally Controlled Mating Box

1. **To ensure a controlled and easy to clean test area, setup a stainless-steel kitchen cabinet of 120 cm x 64 cm x 85 cm as illustrated in Figure 1A.**
    1. Drill one hole at the back of the cabinet just below the ceiling and four sets of four holes into the sides, each with a diameter of 2 cm. Drill the first two sets of four holes, on each side of the box at a height of 7 cm from the bottom of the box and with 12.5 cm in between holes. Drill the other two sets on each side of the box at a height of 35 cm from the bottom.
    NOTE: The four sets of four holes are used for camera power cables and air pump tubing to enter and exit the cabinet. The hole at the back is used for the power cables of a light board.
    2. Build a light board with 18 rows of 40 alternating white and red light-emitting diodes (LED) with 2.5 cm space between each light.Mount the white and red LEDs in a circuit with power supply. Connect each LED in series with a resistor of 560 Ω, 0.25W and 5% tolerance.
    NOTE: The emission wavelengths of the red light spanned from 590-661 nm with a sharp peak at 627 nm. The resulting light intensity in the experimental area is approximately 900 lux with both lights on and 90 lux with only the red lights on; this was measured using a smartphone light meter app.
    3. Attach the light board to the top of the stainless-steel cabinet and pass the power cables through the hole at the back.
    4. Connect the adapter of the white LEDs to a power control timer to allow switching off the white LED during the dark phase of the experiment. Connect the adapter of the red light, which flies are blind to[25], to a regular power supply to keep them on for the whole duration of the experiment.
    5. Fix one 110 cm and two 54 cm long metal brackets, each with a width of 0.5 cm, to the inner sides of the box at a height 50 cm from the bottom of the box. Place a frosted glass diffusion plate (dimensions of 119.0 cm x 54.5 cm x 0.5 cm) on these brackets.
    6. Add three layers of filter paper (120 cm x 50 cm) in between the light board and glass plate to diffuse the light and limit glare on the surface of the mating arenas (described in section 4). Pin two filter paper sheets together on their long edge on 120 cm long wooden rods using magnets (on the side), to stick them to the insides of the metal cabinet; perform this action 3 times.
    7. Attach four fans at the sides of the box to create an air stream that continuously vents the mating box. Attach the first set of 8 cm fans between the light board and glass plate, with the inlet of air at the left side and the exhaust at the right side of the box, to minimize the building-up of heat generated by the light board.
    8. Attach the second set of 12 cm fans 25 cm above the bottom of the cabinet to create an outward air flow that vents the inside of the cabinet and cools it to a stable 26 °C. Attach the fans at the exhaust side to a suction hose and lead the airstream out of the room to prevent re-cycling of the air in the cabinet.
2. Set up two stands (approximately 48 cm tall) mounted with two clamps, one at 28 cm and one at 30 cm from the base of the stand. Fix a webcam onto each of the clamps. Connect the 4 cameras to a computer running the monitoring software.
3. Place A4 sheets underneath each webcam. Use unprinted white sheets or sheets with pre-numbered grid of 7 by 5 squares with 4 cm axes to accommodate mating arenas (described in section 4).
NOTE: An HD webcam camera with 78° wide-angle view and 5-million-pixel resolution can cover an area of 21 cm x 30 cm corresponding to an A4 sheet and monitor between 20 and 35 mating arenas.

2. Fly Rearing and Collection

1. **Place 20 male and 20 female wild-type *Canton-S* flies into fly rearing bottles containing 45 mL rich fly food medium (see section 3) for three to four days. Transfer the same adults three times by first tapping them down and then into fresh bottles.**
    1. Place the bottles in an incubator at 25 °C, and 12 h: 12 h light-dark cycle with lights on at 09:00 (Zeitgeber time (ZT) 0). A new generation will appear about 10 days later.

2. **Anesthetize the resulting newly eclosed flies on carbon dioxide pads for no longer than 5 minutes and collect them into fly food vials using a paint brush.**
    1. Collect virgin (newly eclosed) females and virgin males from the wild-type *Canton-S* stock bottles into 2.5 cm x 9.5 cm fly rearing vials with 6.5 mL of rich fly food medium.

3. Age the flies in same sex groups of 20 flies each in fly rearing vials for 5 to 8 days at 25 °C and 12 h: 12 h light-dark cycle and lights on at 09:00 (ZT 0).
4. Transfer the flies to fresh fly rearing vials on the day before the experiment.

3. Food Medium Preparation

1. **Prepare 1 L of rich fly medium as follows.**
    1. Pour 1 L of tap water in a 2 L glass beaker with a magnetic stir bar and put the beaker on a magnetic hot plate. Keep the stirring off and turn the heating up to 300 °C until boiling temperature is reached.
    NOTE: During the long boiling time in the following steps a proportion of water will evaporate, but together with the added ingredients this protocol results in 1 L of rich fly medium when prepared at room temperature of approximately 22 °C.
    2. Turn on the stirring to 500 rounds per min (rpm) and add the following ingredients to the boiling water: 10 g agar, 30 g glucose, 15 g sucrose, 15 g cornmeal, 10 g wheat germ, 10 g soy flour, 30 g molasses, 35 g active dry yeast. Wait for the yeast to foam vigorously, then turn down the hot plate temperature to 120 °C.
    3. After 10 min turn the hot plate down to 30 °C and let the mixture stir until cooled to 48 °C. Monitor temperature by inserting a thermometer directly into the food.
    4. Dissolve 2 g of p-hydroxy-benzoic acid methyl ester (tegosept,100%) into 10 mL of 96% ethanol. Add this and 5 mL of 1 M propionic acid to the mixture. Stir for 3 min.
    5. Pour the fly food medium into the arenas (described in section 4) to create a 0.3 cm thick layer at the bottom of the arena.
        1. Use a 200 mL glass beaker for pouring. When exact quantities are important, use a 10 mL serological pipette.

2. Prepare fly medium minus yeast exactly as described in step 3.1.1 until 3.1.5, but leave out the yeast in step 3.1.2.
3. Prepare medium with agar with or without peptone by mixing 10 g agar and 35 g peptone in 1 L of boiling water and perform steps 3.1.4 to 3.1.5.

4. Mating Arena Preparation

1. Pierce a hole approximately 0.3 cm in diameter on the upper side of a 3.5 cm x 1.0 cm plastic Petri dish using a heated preparation needle (heated to redness in a Bunsen burner). Alternatively, use a soldering iron.
2. When preparing food medium with odorous compounds, first pipette 30 µL (1% of the final food medium, e.g. Acetic Acid glacial 100%) of desired compound into the dish for half of the experimental dishes. Leave the other half of the dishes empty for comparison.
NOTE: With the set-up described here, a maximum of 140 arenas can be tested at once.
3. Using a 10 mL serological pipette, pour 3 mL of food medium at the bottom of the dish on top of the desired compound. Cover it with a cheese cloth to prevent contamination, and leave the medium to solidify for approximately 1 h at room temperature.
4. Place a lid on the dishes and tape each shut at two sides. Prepare small paraffin film plugs to cover the holes of the dishes by rolling pieces of paraffin film into 0.2 cm thick rolls and then cut them into 0.5 cm segments.

5. Yeast Culture for Odor

1. **Grow dry active yeast on yeast extract peptone dextrose (YPD) agar in a 14.0 cm x 2.06 cm Petri dish. Wear gloves to prevent contamination in this step.**
    1. Prepare YPD agar plates by adding 10 g yeast extract, 20 g peptone, 22 g glucose (0(+)-glucose monohydrate) and 15 g agar (pure) to 1 L of boiling ultrapure water. Layer the bottom of the Petri dish once everything is dissolved and store upside down in the refrigerator at 4 °C, for up to 2 months.
    2. Sprinkle a few grains of dried yeast on a YPD medium plate, let them dissolve. Then streak the medium plate using a sterile loop. Store the plate in a 30 °C incubator overnight. Afterwards, store the culture in the refrigerator for no longer than 1 week.

2. **Prepare YPD liquid medium in 1 L bottles by adding 10 g yeast extract, 20 g peptone, 22 g glucose (0(+)-glucose monohydrate) and a stir bar to 1 L ultrapure water.**
    1. Autoclave for 25 min at 120 °C and 1 bar pressure. Afterwards, store the bottles at 4 °C for up to 2 months until use.

3. **Fit open bottle caps (4.5 cm) with a 0.32 cm thick silicone septum.**
    1. Cut two small holes in the septum to snugly fit barbed bulkhead fittings. Attach the small polyvinyl Chloride (PVC) tubing (diameters: outer 0.8 cm and inner 0.5 cm) to both outlets that exit the bottle and to only one of the inlets entering the bottle. See figure 1B for illustration.
    2. Wrap the fitted caps and tubing in aluminum foil and autoclave for 25 min at 120 °C and 1 bar pressure.

4. **Wear gloves to protect against contamination in this step. Dip a sterile 100 µL pipette tip into one of the yeast colonies from the YPD agar plate (described in 5.1) and drop it into the autoclaved YPD liquid medium bottle.**
    1. Cap this yeast-inoculated YPD liquid medium bottle as well as a YPD medium control bottle (yeast not added) with autoclaved caps mounted with in- and outlet (described in step 5.3). Put both bottles on separate magnetic plates and stir at 100 rpm at room temperature for 24 h before the start of the experiment to allow the yeast culture to grow.
    2. Connect the inlets of both bottles to separate aquarium pumps to supply air to the yeast culture. Make sure to connect the outlet of the experimental yeast bottle to a tube venting the yeast smell out of the experimental room to prevent interference with the experiment.

6. Air Pump Set-up

1. Attach the large PVC tubing (diameters: outer 1.2 cm and inner 0.9 cm) to a pressurized air supply and lead it through two 1 L glass Erlenmeyer flasks filled with activated charcoal to the 800 mL line to purify the air. Use either pressurized air commonly supplied in labs as air supply, or connect the tubes to an air pump (pressurized air was used here).

NOTE: Tubing material should be selected based on the chemical properties of the volatile, and tested to prevent the volatile from sticking to the lining of the tubing (e.g. polytetrafluoroethylene, nylon or stainless steel).

2. **Make two air splitters from 15 mL tubes and three 1,000 µL pipette tips each.**
    1. Make three holes of ~1 cm diameter. First burn two holes, using a heated preparation needle (heated red in a Bunsen burner), adjacent to each other just below the lid of the 15 mL tube. Then make the third hole by removing the bottom of the tube.
    2. Glue the 1,000 µL pipette tips into the holes with the narrow end pointing outward. Cut the end of the pipette tips to allow greater air flow.
3. Connect the large PVC tubing from the outlet of the charcoal-filled Erlenmeyer flask to the pipette tip at the bottom of the 15 mL tube. Add small PVC tubing outlets to the two horizontal pipette tips and lead them towards a control and an experimental bottle.
4. To prevent contamination of the YPD medium with microorganisms, attach the small tubing to a sterile syringe filter (0.45 µm pore size) with a plastic push on bulkhead tubing connector going towards the filter, and a screw on plastic bulkhead connector leaving the filter. Then, attach the tubing to the inlet of the YPD culture bottle (see **Figure 1B**).
5. **To prevent airborne yeast from traveling from the culture flask into the experimental arena, attach a glass tube (6.5 cm long, outer diameter = 0.5 cm and inner diameter = 0.3 cm) to the outlet of each YPD culture bottle using small tubing. Attach PVC tubing to the other side of the glass tube and lead this towards the lower holes (drilled at each side) of the experiment box.**
    1. Fill the tube with glass fiber and autoclave it before use.
6. Add another 15 mL tube splitter (described in section 6.2) to the small PVC tubing, at each side of the experimental box, to get two tubes running into the box at both the experimental side (closest to exhaust of fan air stream, right) and the control side (closest to the inlet of the fan air stream, left).
7. Prepare 8x 25 mL serological pipettes each with 10 outlets to test 80 mating couples at the same time, *i.e.* 40 for each air condition.
    1. Burn 10 holes of ~0.8 cm diameter, 2 cm apart into the pipette.
    2. Cut the outer part of a 1 mL syringe into one small (2.5 cm) and one big (5 cm) outlet.
    3. Glue these outlets into the holes with hot glue.
    4. Wrap a small band of plastic paraffin film around the end of the outlets and attach a 1,000 µL pipette tip. Diameter of the tip opening is 0.1 cm. Use clean tips for each experiment.
8. Attach two serological pipettes, using a T-splitter with outer diameter ≥0.5 cm and short pieces of small PVC tubing, to each of the two outlets at both sides. Tape the pipettes flat on the white paper sheet (under the cameras in the steel box).
9. Using an air flow monitor, set the air flow so that air velocity at the exit of the 1000 µL pipette tip is 0.5 m/s. This corresponds to an air flow of 0.0017 L/s per tip.

7. Monitoring of Mating Behavior

1. Use a mouth pipette (as described in reference[26]) to place one experimental female into a small Petri dish (described in section 4) at 15:00 o'clock (ZT 6) and give her 1 h to acclimatize to the mating arena.
2. **Set up the experimental box (described in section 1) as follows:**
    1. Turn on the lights, *i.e.* white light LEDs on a 12h :12 h light-dark cycle connected to a timer that switches on the light at 09:00 (ZT0) and continuous red light LEDs to allow monitoring of the flies during the dark phase of the experiment. Turn on the fans to limit heating up of the cabinet by the light source and to ensure excess odors are vented outside the test area.
    2. Connect webcam cameras to a computer and start them with the monitoring software for picture monitoring.
    3. For each camera, set the focus, brightness, and zoom in the monitoring software.
        1. Right-click on the camera screen, open "camera properties," and unclick "automatic focus." Adjust the "focus" to clarify of the grid or any written words on the paper sheet. If necessary, change the "brightness" and "zoom."
    4. Set the program of the monitoring software to capture 1 picture every 2 min. Right-click on each camera screen and choose "edit camera" and then the "Actions" option. Click to start the actions "At regular intervals" and change the time to "2 minutes." Choose "Take Photo" for select actions to perform and finally click on "ok."
    5. Right-click on each camera screen and select "start monitoring."
3. After 1 h (at ZT 7), transfer a wildtype male to the Petri dish using the mouth pipette, place the dish on the A4 paper sheetsunder the webcam cameras, and click "start monitoring" for 24 h. For the air pump experiment, place the dish in such a way that a pipette outlet is connected to the entrance hole of the mating arena.
4. **To analyze the mating behavior of the couple, do the following.**
    1. Select and open all pictures in an image viewing software and page through them in chronological order.
    2. Write down the date, experiment number, dish number, and start time into a spreadsheet within the same row. Take the start time of each arena from the moment that it is placed under the webcam camera. Record the time stamp from the pictures.
    3. Mark the start time of each copulation in the same row into the spreadsheet. Count a mating as an incident when the male has mounted the female and the couple remains moderately stationary and in the same posture for at least five consecutive frames (10 min).
    NOTE: This criterion is based on the reported length of copulation, ranging from 12 to 27 min in *D. melanogaster*, and the observation that copulations of 10 min and over are fertile[27,28].
    4. Count the number of copulations for each row in the spreadsheet to determine the mating frequency. Alternatively, subtract the start time of the experiment from the time of first mating for each row as a measure of mating latency, or subtract the time of the first mating from the time of the second mating as a measure of remating latency.
        1. To calculate the remating latency, make sure to define the dates of the first and the second mating as consecutive days in the spreadsheet software.

5. Analyze the data with mixed effects models, assuming normal distribution of the data and including the date of the experiment as a random factor, using a statistical software (see the table of materials) to determine the statistical significance of the independent variables–food medium, air type, and interaction–as previously described[5].
6. Select the best explaining model by performing the backwards elimination of non-significant independent variables using log-likelihood ratio tests and the associated Akaike information. After running the model, visually inspect the data residuals to confirm normality. Confirm the homogeneity of variances using the Levene's test. In the case of unequal homogeneity, square-root transform the data.

Representative Results

Using this continuous assay, mating behavior, and mating frequency in specific, can be determined under experimental environmental conditions. To control environmental conditions, we transformed a stainless-steel kitchen cabinet into a test area, with its own light source and diffusion, which ensures a high abundance of light and a minimum amount of glare from the top of the mating arenas (**Figure 1A**). The inner test area is completely encased by stainless steel and glass, which allows for cleaning with organic solvents, such as hexane or ethanol. Additionally, the cabinet is equipped with holes that act as inlets for tubing, bringing volatile cues from the pressurized air system (see **Figures 1A** and **1B**). The pressurized air system, adjusted for yeast odors, consists of an airflow guided through a liquid yeast culture before entering the test arenas through 4 pipette splitters with 10 outlets each (**Figure 1C**). The whole system is airtight and fitted with several particle filters, both before and after entering the yeast culture, to minimize contamination with confounding odors (**Figure 1B**).

To demonstrate the use of this assay, we tested whether volatile cues from a yeast culture can influence mating behavior. Air was bubbled through a liquid yeast culture for 24 h, and the air outlets were placed in the entrance of each mating arena (see **Figure 2A**). Half of the mating arenas contained fly food with yeast (Food + yeast), and the other half contained fly food without yeast added (Food - yeast). A wildtype male and female were exposed to the odors coming from the external yeast culture, and their mating frequency was recorded. To determine which variables are necessary to explain the graphed results, we ran mixed-effects models, either including or excluding the independent variables of food medium, yeast air, and an interaction of the two. The data in **Figure 2B** is best represented by a model including the independent variables of food medium ($p = 0.001$) and yeast air ($p = 0.061$), but there is no explaining interaction effect. Even though the yeast air variable is not significant in this full data set, it is necessary to explain the results. Analysis of yeast air separated for food medium shows that a mating couple does not respond to yeast odors when there is no yeast present in the food medium (air: $p = 0.992$), but they do increase their mating frequency in yeast air when yeast is also added to the food medium (air: $p = 0.018$). Together, these results demonstrate the applicability of the pressurized air system to test the influence of environmental odors in combination with food medium conditions.

We also illustrate how the pressurized air system can be bypassed by adding environmental chemical cues directly to the test arena. To demonstrate which specific yeast compounds affect mating frequency, we tested the hypothesis that the amino acid content of yeast is necessary for its effect on mating by placing a dose of peptone (hydrolyzed proteins) corresponding to the amino acids supplied by the yeast in the agar substrate lining the mating arena. We also tested the necessity of acetic acid, one of the major volatile fermentation products of yeast, to increase mating frequency. This was done by adding acetic acid directly to the food medium. A wildtype male and female were tested in arenas containing agar or agar with peptone, with or without acetic acid directly in the food medium (**Figure 3B**). This makes for a very simple food medium and a poor environment; therefore, the average mating frequency is also decreased as compared to Figure 2B. The data in **Figure 3B** is best represented by a model including the independent variables of food medium ($p = 0.002$), acetic acid air ($p = 0.001$), and the interaction of the two ($p = 0.022$). Female receptivity increases upon the presence of acetic acid, but only in the condition where peptone is present in the medium. This shows that flies need to simultaneously sense amino acids and acetic acid to increase their mating frequency (**Figure 3B**). This demonstrates that adding odorous compounds directly to the test arena can influence mating behavior and that those influences can be detected in very simple environmental conditions.

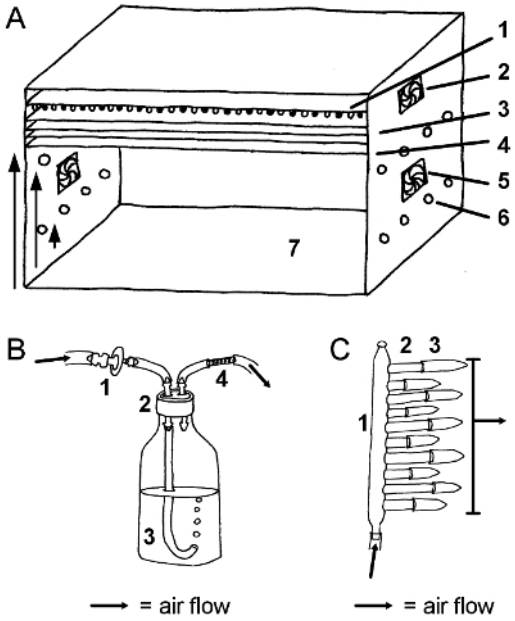

Figure 1: Diagram of the experimental box and pressurized air system with yeast. (**A**) Schematic illustration of the environmentally controlled mating box described in section 1. Description of the annotated numbers and arrows: 1. light board with alternating white and red lights; 2. small fan; 3. 3 layers of filter paper, each layer consisting of two filter-paper sheets; 4. glass diffusion plate resting on brackets attached to 3 sides of the box; 5. big fan; 6. holes for tubing and cables; 7. experimental area; large arrow, 50 cm to the glass plate; middle arrow, 35 cm height for the cable holes; and small arrow, 7 cm height for the tubing holes. (**B**) Schematic illustration of the liquid yeast culture with airflow, as described in sections 5, 6.4, and 6.5. Description of the annotated numbers: 1. disposable filter unit; 2. cap with silicone septum and out- and inlets; 3. liquid medium; and 4. glass tube with glass fiber. (**C**) Schematic illustration of the air outlets as described in section 6.7. Description of the annotated numbers: 1. serological pipette; 2. tubing cut from 1 mL syringe, and 3. 1,000 µL pipette tip. Please click here to view a larger version of this figure.

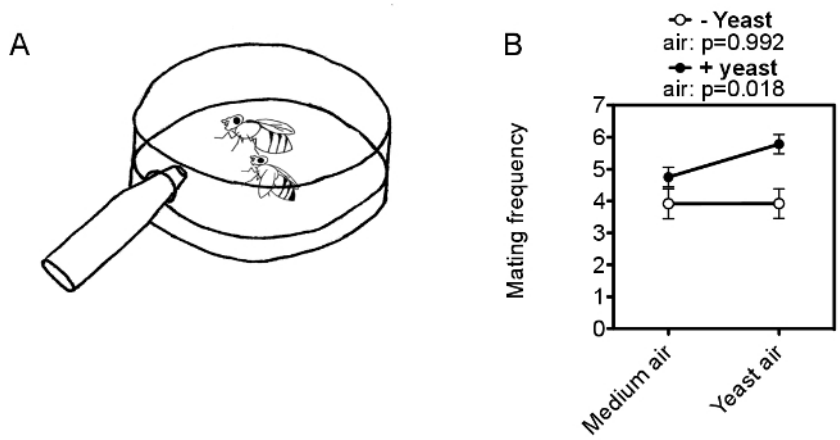

Figure 2: Yeast odor increases female receptivity in the presence of yeast in the food substrate. (**A**) Schematic illustration of a mating arena with one male and one female and a pipette tip from the air outlet in Figure 1C entering through the entrance hole. (**B**) Graphical presentation of the response in mating frequency of a *Canton-S* mating couple to yeast odor with and without yeast in the fly food medium (Food – yeast: medium air n = 12, yeast air n = 13 and Food + yeast: medium air n = 24, yeast air n = 23). Line graph with SEM error bars and statistical output of mixed-effects models with air as the independent variable and the date as a random variable for each food medium independently. The main statistical model includes food (p = 0.001) and yeast air (p = 0.061). Adapted from reference[5]. Please click here to view a larger version of this figure.

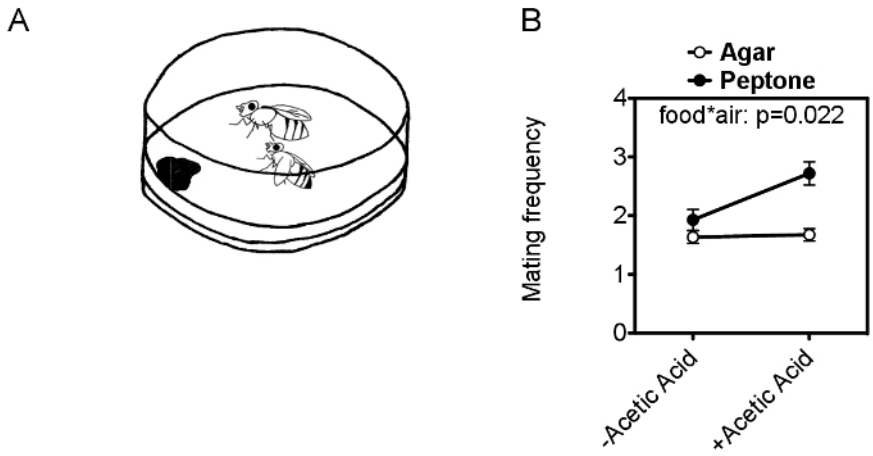

Figure 3: Acetic acid in fly food substrate increases female receptivity in the presence of peptone. (**A**) Schematic illustration of a mating arena, with fly food medium containing acetic acid and a plastic paraffin film plug closing the entrance hole. (**B**) A graphical presentation of the mating frequency of a *Canton-S* mating couple in response to acetic acid either on agar or peptone medium (agar: -acetic acid n = 52, +acetic acid n = 40 and peptone: -acetic acid n = 28, +acetic acid n = 25). Line graph with SEM error bars and the statistical output of the mixed-effects model with food medium (p = 0.002), acetic acid air (p = 0.001), and food*air (p = 0.022) as independent variables and the date as a random variable. Adapted from reference[5]. Please click here to view a larger version of this figure.

Discussion

This protocol describes an assay to test mating behavior over 24 h while continuously controlling the environmental cues that a mating couple is hypothesized to use to determine mating frequency. It is possible to increase the mating frequency in response to yeast air delivered through a pressurized air system when the medium contains yeast as well (**Figure 2B**). Additionally, a similar response in mating frequency can be observed with a simplified food medium containing only agar, peptone, and acetic acid odor directly in the medium (**Figure 3B**)

With the experiments demonstrated here, conclusions can only be drawn on the general mating behavior of the couple, since both sexes are exposed to the same environmental conditions. However, we know from previous research that 47% of the variation in mating frequency is determined by the female, while the male contribution only accounts for 11% of the variation[20]. Therefore, most of the changes in mating frequency observed are likely a result of female sexual receptivity. Increased males courtship still leaves the female to accept or reject mating, as adult *D. melanogaster* females can successfully deflect mating attempts[29]. For firm conclusions and to specifically attribute differences in mating frequency to female sexual receptivity, it is necessary to test additional mating couples where the genotype of the female is varied but that of the male is kept constant.

This protocol has demonstrated two ways to deliver odorous compounds to a mating couple, either with a pressurized air system or directly into the food medium. The pressurized air system has the advantage that any effect can be attributed to the compounds that are delivered through the air, while this cannot be concluded when the compounds are put directly into the food medium. On the other hand, when no effect is found with the pressurized air system, it does not automatically mean that the cue does not affect behavior. It could also mean that the compound is not efficiently delivered through the pressurized air system. The composition of the air at the outlet of the air delivery system can be analyzed by placing a hydrocarbon filter and analyzing the trapped air content with gas chromatography coupled with mass spectrometry. The pressurized air system is a good assay to test compounds that can be easily made airborne over a longer range. Less volatile compounds might have to be put directly into the food medium. Another disadvantage of the pressurized air system is the effect air velocity can have on fly behavior. Flies stop moving when the air velocity is too high (above 0.7-1.6 m/s)[30]. Additionally, the pressurized air system can render a simple, low-quality environment intolerable by drying out the food medium. In both cases, the flies might not perform equally well, and no conclusions can then be attributed to the specific compounds tested.

Several steps are essential during preparation for the optimal running of these assays. The first step that requires attention is preparation of the medium. It is important that the medium, including odorous volatile compounds such as acetic acid, is prepared on the day of the experiment and not sooner in order to avoid evaporation. Also, the medium needs to harden on a surface with no extra airflow (avoid using fume hoods for this), because airflow can stimulate the evaporation of the odor. The second step that requires special care is the establishment of the pressurized air system. The airflow needs to be high enough to gently bubble the yeast culture without transferring any fluid to the arena.

This protocol demonstrates a behavioral assay with yeast odors in combination with mating behavior. However, this system can be applied to any type of odor, as well as to other types of behaviors. To use this system for other odors, it is necessary to adjust the airflow and odor medium to optimize the transfer of the compounds to the dishes. However, in general, any compound that can be transferred by air can be tested with this system. In addition, any type of behavior, in both males and females, can be tested, either by using the same type of dishes or by adjusting the tubing to reach and connect to bigger or smaller test areas. Additionally, when more detailed behaviors are tested, the frame rates and resolutions of the cameras used need to be reconsidered. In any case, if both experiments with and without the test odor are run at the same time and with the same air source, any response to the environmental cue can be detected, regardless of changes in pressure or concentration

from one experiment to the other. Lastly, the assay demonstrated here can be extended for at least another LD cycle (up to 48 h), as long as the food supply does not dry out.

Disclosures

The authors have no competing interests to disclose.

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